The Influence of Acetic Acid Concentration on the Extractability of Collagen from the Skin of Hybrid Clarias sp. and Its Physicochemical Properties: A Preliminary Study

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Abstract

Keli, cultured hybrid catfish of *Clarias* sp. (*Clarias gariepinus* ×*C. macrocephalus*) is an under-utilized and abundant freshwater fish in Malaysia. Type I pepsin soluble collagen (PSC) was isolated from the skins of this fish species. Different concentrations of acetic acid solution used as extracting medium and the corresponding effect on the yield of extraction were studied. The maximal yield of PSC was achieved when the acetic acid used was at 0.7 M, whilst a significant drop in the yield was observed beyond this concentration. In the physicochemical properties of catfish skin collagen, the amide A, B, I, II, and III regions in Fourier transform infrared measurements were shown in the peaks of 3429 cm^{-1}, 2929 cm^{-1}, 1652 cm^{-1}, 1551 cm^{-1}, and 1240 cm^{-1}, respectively. There was also a close similarity in the amino acid composition of *Clarias* sp. skin collagen with other sources of collagen. The preliminary results showed that *Clarias* sp. can be exploited effectively for collagen extraction.

Keywords

Acetic Acid Concentration; Collagen; Freshwater Fish; FTIR; Amino Acid Composition

Introduction

In recent years, collagen has been employed in the food, pharmaceutical, cosmetic, and medical industries (Sadowska et al., 2003). This popular biomaterial is the most abundant animal derived protein and presents in almost all tissues and organs of vertebrates, including skin, cartilage, bone, muscle, blood vessels as well as various supporting tissues (Yuna et al., 2010). Though collagen can be found in all animal parts, Nakamura et al. (2003) reported that type I collagen is especially concentrated in skin-associated tissues and bones. Most commercial collagens, however, are derived from bovine hide, pig skin, and chicken wastes, creating much anxiety among health-conscious consumers for the past decades due to the outbreaks of bovine spongiform encephalopathy (BSE), foot-and-mouth disease, and avian flu (Ali, 2010). Additionally, Hindus do not consume cow-related products whilst Muslims regard all pork-related products to be non-halal and are not permitted to be consumed (Herpandi and Adzitey, 2011). Therefore, alternative sources, mainly those of marine sources have been paid increasing attention as the potential replacement for mammalian or land animals collagen.

Numerous researches are intensive in the functional properties of marine collagens, especially extraction from fish skin since it represents an important source of highly soluble collagen (Giménez et al., 2005). These include skin of yellowfin tuna (Woo et al., 2008), skin of striped catfish (Singh et al., 2010), skins of young and adult Nile perch (Muyonga et al., 2004), skin of grass carp (Wang et al., 2009), skin of Baltic cod (Sadowska et al., 2003), skin of bigeye snapper (Kittiphattanabawon et al., 2005), skin of brown backed toadfish (Senaratne et al., 2006), and skin of skate (Hwang et al., 2007). Fish collagen is undoubtedly able to serve as a viable alternative for safer and consumer-friendly collagen. Up to now, most of the studies have utilized by-products such as skins, bones, and fins during processing of fishes as the raw materials for collagen extraction. Fish filleting industry in Malaysia, however, is not as common as in Japan, Thailand, and China which are among the largest surimi producers worldwide. In fact, the Department of Fisheries Malaysia is currently facing a major

constrain due to lower conversion ratio when freshwater species are utilized in the industry which results in higher producing cost in contrast to deep sea species.

Demand for freshwater fishes in Malaysia is only meant for daily consumption so far, resulting in their low commercial values and indirectly hampering the large-scale development of fish farming industry in Malaysia. This is due to less exploitation of these natural resources and their conversion into value-added products which are not limited to only food industry is expected to yield additional income, thus offering economic benefits to both the fisheries industry and local fishermen. Locally known as *Keli*, cultured hybrid catfish of *Clarias* sp. (*Clarias gariepinus* ×*C. macrocephalus*) has been a staple fish in Malaysia. Catfish is a good source of protein with a considerable amount of collagen exists in the muscles and skins (Sivakumar et al., 2000). In spite of that, commercial value of this hybrid catfish is much lower as compared to other cultured fishes due to its abundancy in the market. As reported by Anon (2011), cultured catfish production in Malaysia showed significant improvement in recent years by 7 folds from 7, 158 tons in 1999 to 81,041 tons in 2009. Hence, it is as an interesting attempt to boost up the commercial value of this cultured catfish by utilizing the skin as the raw material for collagen extraction.

Collagen extraction is often carried out by direct extraction with organic acids such as acetic acid, chloracetic acid, citric acid, or lactic acid (Skierka and Sadowska, 2007). Though inorganic acid such as hydrochloric acid has also been employed for extraction of collagen, its efficiency is lower than that of the organic acids (Wang et al., 2009). Among all, acetic acid is regarded as the most promising solvent in extracting collagen from different sources (Muyonga et al., 2004; Kittiphattanabawon et al., 2005; Wang et al., 2009; Singh et al., 2011). The solubility of collagen in acid solution plays a key role in the extraction efficiency. According to Giménez et al. (2005), increase in H^+ ions aids the access of water to collagen fibres. The water is held in by either electrostatic swelling (electrostatic forces between charged polar groups) or lyotropic hydration (hydrogen bonding between uncharged polar groups and negative atoms). Consequently, solubilization of collagen in the extracting medium strongly depends on the concentration of acid used which will affects its swelling properties.

The literature on the extraction of collagen by different acid concentration and their influence on the yield are limited. There is also no information on the extraction of native collagen from the skin of hybrid *Clarias* sp. Therefore, the aims of this research were to compare the yield of collagen extracted from catfish skin with different acid concentration and to carry out a preliminary investigation on the physicochemical properties of the resulting collagen.

Materials and Method

Materials

Cultured catfish (hybrid of *C. gariepinus x C. macrocephalus*) were purchased from a local wet market in Parit Buntar, Perak. Upon arrival at the laboratory, the fishes were killed, dissected, deboned and the skins were cleaned of adhering tissues before being cut into small pieces (1 cm × 1 cm) and then washed with distilled water and kept frozen at -20°C prior to collagen extraction.

Chemicals

Commercial pepsin from porcine gastric mucosa, sodium hydroxide, and acetic acid were purchased from Merck Sdn. Bhd. (Malaysia). All other chemicals used were of analytical grade.

Isolation of Skin Type I Collagen

1) Extraction of Pepsin Soluble Collagen (PSC)

All procedures were performed as previously described by Wang et al. (2009) and Kittiphattanabawon et al. (2005) with slight modifications. The extraction process was carried out at 4°C. To remove non-collagenous proteins, the skins were mixed with 0.1 M NaOH at a sample to alkali ratio of 1:20 (w/v). The mixture was stirred for 6 hr. The NaOH solution was changed every 2 hr. The sample was then washed thoroughly with excessive distilled water until the pH was neutral or slightly basic. Deproteinised skins were defatted with 10% butyl alcohol with a sample to alcohol ratio of 1:20 (w/v) for 24 hr. The alcohol solution was changed every 8 hr. Defatted skins were then washed with cold water and subjected to collagen extraction by aqueous acetic acid. They were actively stirred in acetic acid with varying concentration (0.1 M, 0.3 M, 0.5 M, 0.7 M, and 0.9 M) containing 1.5% (w/w) pepsin for 24 hr to extract pepsin soluble collagen. The viscous collagenous material was separated from the insoluble components by high speed centrifugation at 20,000

× g for 40 mins. The soluble collagen solution was obtained from the supernatant. The collagen was precipitated by adding NaCl to a final concentration of 0.8 M. Resulting sediment was collected by centrifugation at 20,000 × g for 30 mins. To further purify the collagen, it was re-dissolved in minimal amount of acetic acid, dialyzed against 0.1 M acetic acid, followed by distilled water and lyophilized. The freeze-dried product was designated as pepsin soluble collagen (PSC).

(1)

2) Collagen Yield Measurement

The yield of pepsin soluble collagen from the skin of *Clarias sp*. was calculated using Eq. (1):

$$Yield\ of\ collagen\ (\%) = \frac{Weight\ of\ collagen\ (g)}{Weight\ of\ skin\ (g)} \times 100\% \quad (1)$$

Amino Acid Analysis

The freeze dried products were hydrolyzed in inert atmosphere with 6 M HCl containing 1% phenol at 110ºC for 24 hr. The hydrolysates were then dried under vacuum. This was followed by derivatization, drying, and dilution with sample diluents. The amino acids derivative samples were analyzed by high performance liquid chromatography (HPLC) and compared against the standard amino acids which were analyzed prior to these. The area under the peak of each amino acid in chromatogram was calculated and compared with that of the standard and reported as number of residue per thousand amino acids content.

Fourier Transform Infrared Spectroscopy (FTIR)

FTIR spectra were obtained from discs containing 2 mg collagen in approximately 100 mg potassium bromide (KBr). Infrared spectra were obtained in the range between 4000 and 500 cm^{-1} using an infrared spectrophotometer (Model-Shimadzu Scientific Instruments' IR-Prestige-21, Thermo Fisher Scientific, Malaysia).

Results and Discussion

Yield of PSC from the Skin of Clarias sp. with Different Concentration of Acetic Acid

Acid extraction with pepsin digestion is a common method for collagen extraction nowadays. As described by Skierka and Sadowska (2007), the enzyme (pepsin) removes only the non-helical ends of the collagen. Not only the physico-chemical properties of collagen are altered, non-collagenous proteins are also hydrolyzed, thus it is possible to expect an increase in the collagen solubility after enzymatic treatment. With great extractability towards collagen, acetic acid has been frequently used as a solvent for collagen extraction (Cheng et al., 2009). This was supported by the findings of Skierka and Sadowska (2007) and Cheng et al. (2009) who stated that the extraction of collagen from animal tissues through inorganic acid (e.g. hydrochloric acid) resulted in lower efficiency and yield than the organic acids. Fig. 1 shows the effect of different concentration of acetic acid on the yield of PSC extracted from the skin of *Clarias* sp. Yield of PSC increased with the increment of acetic acid concentration to 0.7 M. However, a reverse trend was observed beyond this concentration. The highest yield was achieved when 0.7 M acetic acid was used as the extracting medium and PSC as much as 26.69±0.97% was extracted. For higher concentration of acetic acid particularly at 0.9M, the yield of PSC was found to be lower which was 20.35±0.75 %, significantly ($P<0.05$) lower than that of 0.7 M. The pattern of the yields obtained was almost similar to that reported by Wang et al. (2009) in which the yields of acid soluble collagen (ASC) extracted from grass carp skins were found to be in an increasing manner with the increment of acetic acid concentration to 0.5 M, and thereafter decreased.

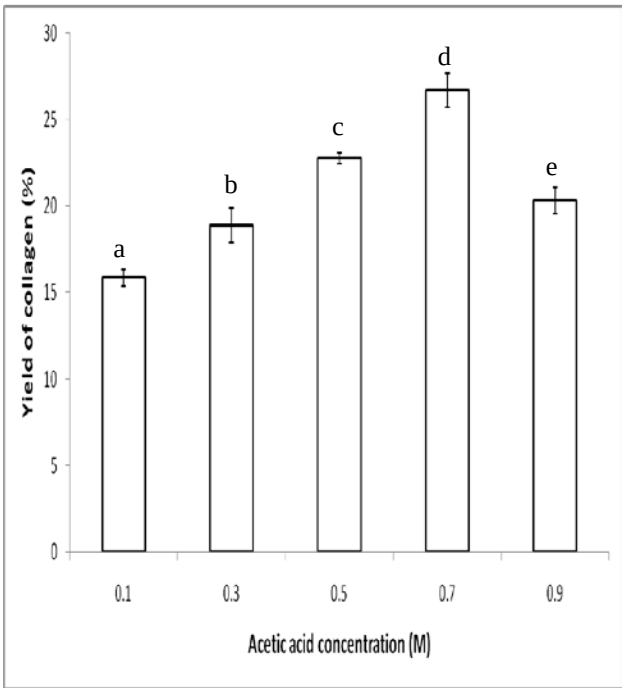

FIG. 1 YIELD OF COLLAGEN AT DIFFERENT CONCENTRATION OF ACETIC ACID AS EXTRACTING MEDIUM (THE COLUMN WITH THE SAME ALPHABET LETTER WAS NOT SIGNIFICANTLY DIFFERENT ($P>0.05$))

Difference in the yield obtained through different

concentration of acetic acid employed was probably due to different solubility of collagen in the acidic extracting medium. Among the concentrations of acid tested, 0.1 M was the least effective solvent for PSC extraction from the skins. During the 24 hr of extraction, only about 16 % of collagen was dissolved. The amount of dissolved collagen was increased when the skins were incubated in higher concentration of acetic acid. Incomplete solubility of the skins suggests that inter-molecular cross-links are still present in collagen molecules. The initial stage of solubilisation of collagen is the hydration of fibrous collagen which proceeds by exposure to acids (Skierka and Sadowska, 2007). Modification of the electrostatic interaction and structure of this protein might occur along the changes in acid concentration since pH value is in charge of the charge density of protein (Verheul et al., 1998).

A very low pH value of the extracting medium, as in the case of 0.9 M (pH 2.39) would result in reduction of water absorption of collagen. Skierka and Sadowska (2007) mentioned that at very low pH, positively charged amine groups of proteins will form bonding with anions (refers to CH_3COO^- for acetic acid aqueous solution), leading to weaker electrostatic repulsive forces between the one-nominal charged group. This will then results in tightening of the structure of collagen fibres and the ability to form bonding with water reduces, thus solubility of collagen in the medium decreases. In addition, Wang et al. (2009) recently stipulated that collagen is denatured at extremely low pH value as collagenous fibres start to shrink at pH around or below 2.0, making protein hydration impossible. These explain the observations in this study that beyond the concentration of 0.7 M, a significant decrease in the yield of PSC was obtained.

Amino Acid Composition of Clarias Sp. Skin Collagen

Table 1 shows the amino acid composition of the pepsin-soluble collagen extracted from the skin of hybrid catfish of *Clarias* sp. The composition was expressed as amino acid residues per 1000 total amino acid residues. Since collagen is triple helical in nature with the characteristic amino acid of $(Gly\text{-}Pro\text{-}Hyp)_n$ (Singh et al., 2011), glycine (Gly) was the most abundant compound with the amount of 234 unit of the total amino acids present in PSC extracted from *Clarias* sp. Even though most of the characterization studies of collagen reported glycine content of approximately 30% of the total amino acids (Kittiphattanabawon et al., 2005; Singh et al., 2011), result in present study is almost similar to the characteristics of collagen isolated from skins of Nile perch as reported by Muyonga et al. (2004) where the glycine content was in the range of 21 – 22%, which was slightly lower than 1/3 of total amino acids. There was also relatively high content of proline while the contents of tyrosine, isoleucine, and methionine were low. This was consistent with the amino acid compositions of collagen reported in literature (Sivakumar et al., 2000; Nagai et al., 2001; Senaratne et al., 2004). Absence of cysteine in collagen obtained from this hybrid species also emphasizes the presence of type I collagen.

TABLE 1 AMINO ACID COMPOSITION OF FISH SKIN COLLAGEN OF HYBRID CLARIAS SP. (AMINO ACID RESIDUES PER 1000 TOTAL AMINO ACIRESIDUES)

Amino acid		
Aspartic acid	Asp	51
Serine	Ser	39
Glutamic acid	Glu	91
Glycine	Gly	234
Histidine	his	152
Arginine	Arg	38
Threonine	Thr	104
Alanine	Ala	42
Proline	Pro	106
Cystine	Cys	0
Tyrosine	Tyr	7
Valine	Val	26
Methionine	Met	16
Lysine	Lys	36
Isoleucine	Ile	14
Leucine	leu	25
Phenylalanine	Phe	19

FTIR Analysis

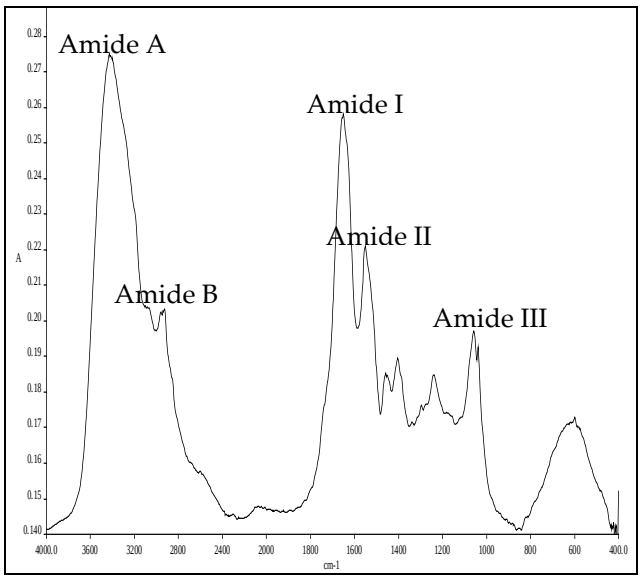

FIG. 2 FOURIER TRANSFORM INFRARED SPECTRA OF TYPE I COLLAGEN (PSC) FROM SKIN OF CLARIAS SP.

The infrared spectra of PSC and the major peaks with their corresponding assignments were shown in Figure

2 and Table 2. FTIR spectra obtained in present preliminary study were similar to those of collagens from other fish species (Muyonga et al., 2004; Singh et al., 2011).

The amide a band of this collagen was found at a wavenumber of 3429 cm^{-1}. According to Abe and Krimm (1972), amide A band is associated with the N-H stretching frequency. A free N-H stretching vibration is expected to occur in the range of 3400 – 3440 cm^{-1}, but Doyle et al. (1975) mentioned that when the NH group of a peptide is involved in hydrogen bond, the position might shift to a lower frequency, usually around 3300 cm^{-1}. The amide I band, with characteristic frequencies in the range of 1600 – 1700 cm^{-1}, was mainly associated with the stretching vibrations of carbonyl groups along polypeptide backbone (Singh et al., 2011). In addition, it was also a sensitive marker of peptide secondary structure (Pati et al., 2010). The peaks of amide I and amide II of *Claris* sp. (1652 cm^{-1} and 1551 cm^{-1}, respectively) were at higher frequencies than those of Catla (1643 cm^{-1} and 1558 cm^{-1}, respectively) (Pati et al., 2010), and striped catfish (1649 cm^{-1} and 1551 cm^{-1}, respectively) (Singh et al., 2011). These indicated that *Clarias* sp. had a higher degree of molecular order than the later since the shift of these peaks to higher frequencies was related to an increase in the molecular order (Payne and Veis, 1988). The ratio of absorption intensity between 1240 cm^{-1} (amide III) and 1551 cm^{-1} (amide II) band was approximately equal to 1.0, which confirms the triple helical structure of collagen from the skin of *Clarias* sp. (Pati et al., 2010; Singh et al., 2011).

TABLE 2 FOURIER TRANSFORM INFRARED SPECTRA PEAK LOCATIONS AND ASSIGNMENT FOR TYPE I COLLAGEN (PSC) FROM *CLARIAS* SP. SKIN

Region	Peak wavenumber (cm^{-1})	Assignment	References
Amide A	3429	NH stretching	Sai and Babu, 2001
Amide B	2929	CH_2 asymmetrical stretching	Abe and Krimm, 1972
-	2075	CH_2 symmetrical stretching	Abe and Krimm, 1972
Amide I	1652	C=O stretching, hydrogen bonding coupled with COO-	Payne and Veis, 1988
Amide II	1551	NH bending coupled with CN stretching	Jackson et al., 1995
-	1459	CH_2 bending	Jackson et al., 1995
-	1403	COO- symmetrical stretching	Jackson et al., 1995
Amide III	1240	NH bending coupled with CN stretching	Jackson et al., 1995
-	1059	C-O stretching	Jackson et al., 1995
-	1039	C-O stretching	Jackson et al., 1995
-	599	Skeletal stretching	Muyonga et al., 2004

Conclusion

Pepsin soluble collagen (PSC) isolated from the skins of cultured hybrid catfish of *Clarias* sp. was classified as type I based on this present preliminary study with slightly different amino acid compositions. It was found that a large amount of collagen could be obtained from the skins of this particular freshwater fish. In the case of the effects of acetic acid concentration, increment of concentration was found to be in favour of improving the yield of collagen extracted. Amount of collagen isolated was increased with the increment of acetic acid concentration to 0.7 M, and thereafter decreased. These findings were helpful for the development of collagen from the under-utilized freshwater fish where they indicated that *Claris* sp. skins exhibited the potential to serve as one of the alternatives of mammalian collagen.

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